REMARKS

This Amendment is filed in response to the Office Action dated October 9, 2007, and is respectfully submitted to be fully responsive to the rejections raised therein. Accordingly, favorable reconsideration on the merits and allowance are respectfully requested.

In the present Amendment, claims 1-12 have been amended to replace "derivative" with "compound".

Claims 2-7 and 10-12 have been amended by changing "A" to "The".

Claim 8 has been amended by changing the expression "for the treatment of allergic diseases" to "for the treatment of type IV allergic dermatitis". Support for the amendment can be found in the specification on pages 69-73 (Evaluation Examples 1 and 2), including on page 72 at lines 3-7, and on page 73 at lines 9-14, for example.

Claim 9 has been amended by changing the expression "for the treatment of pruritus" to "for the treatment of pruritus induced by substance P". Support for the amendment can be found in the specification on pages 73-75 (Evaluation Example 3), including on page 75 at lines 4-9, for example.

Claims 13-18 have been newly added and depend directly or indirectly from claim 1.

Accordingly, no new matter has been added. Entry of the Amendment is respectfully submitted to be proper. Upon entry of the Amendment, claims 1-18 will be all the claims pending in the application.

I. Response to Rejection Under 35 U.S.C. § 112, First Paragraph

Claims 8 and 9 are rejected under 35 U.S.C. § 112, first paragraph, as allegedly lacking enablement for the treatment of any allergic disease and/or for the treatment of pruritus.

Particularly, the Examiner takes the position that Applicants' specification is enabling for a pharmaceutical composition for the treatment of dermatitis, but the specification does not enable any person skilled in the art to which it pertains, or with which it is mostly connected, to use the invention commensurate in scope with Claims 8 and 9.

Applicants traverse and respectfully request withdrawal of the rejection in view of the following remarks.

Applicants submit that the following remarks and cited articles provide proof that one skilled in the art would be able to make and use the claimed invention using the specification as a guide. The evidence provided need not be conclusive, but merely convincing to one skilled in the art. As discussed below, Applicants have provided scientific articles as convincing evidence showing what one skilled in the art knew at the time of filing the application.

First, without conceding to the merits of the rejection, Claim 8 has been amended by changing the expression "for the treatment of allergic diseases" to "for the treatment of type IV allergic dermatitis". Further, Claim 13 has been newly added and is directed to a method of using the uracil compound to treat type IV allergic dermatitis. Support for amended claim 8 can be found in the present specification in Evaluation Examples 1 and 2. Evaluation Examples 1 and 2 demonstrate that the uracil compounds of the present invention are effective in the treatment of type IV allergic inflammation. Applicants submit that the Evaluation Examples 1 and 2 disclosed in the present specification demonstrate that the uracil compounds of the present invention are effective in the treatment of type IV allergic dermatitis. In view of the above arguments and in view of the fact that the Examiner concedes that the specification is enabled for a pharmaceutical composition for the treatment of dermatitis, Claims 8 and 13 meet the enablement requirement.

Next, the specification provides enablement for the scope of Claim 9. Claim 9 has been amended by changing the expression "for the treatment of pruritus" to "for the treatment of pruritus induced by substance P". Support for the above amendments can be found in the pharmaceutical data set forth in Evaluation Example 3 of the present specification. Evaluation Example 3 demonstrates that the uracil compound as recited in the claim, inhibits the itch-related behavior induced by substance P.

Also, Articles (a) - (c), provided herewith, provide evidence of what was well known to one skilled in the art, at the time of filing the present application. Specifically, the articles teach that substance P induces itch, and that substance P is involved in hemodialysis-associated pruritus and the pruritus of atopic dermatitis and psoriasis. Copies of Articles (a) to (c), as listed below, are being submitted concurrently with the present Amendment.

Article (a): Andoh et al. *The J. Investigative Dermatology* **2001**: 117(6), 1621 - 1625;

Article (b): Tobin et al. *J. Allergy Clin. Immunol* **1992**: 90, 613 - 622; and

Article (c): Kuraishi et al. *Eur. J. Pharmacol.* **1995**: 275, 229 - 233.

Articles (b) and (c) are referenced in Evaluation Example 3 in the present specification, and were previously submitted in Applicant's Disclosure Statement, filed April 27, 2005.

According to the teachings of Article (a), when substance P is applied to the skin, it elicits an itch sensation in human subjects. Article (a) further teaches that substance P is involved in hemodialysis-associated pruritus and the pruritus of atopic dermatitis and psoriasis.

Article (b) teaches (in the Abstract at lines 13 to 15) that an increased density of fibers was observed for all markers except Neuropeptide Y and Tyrosine hydroxylase in biopsies from skin with atopic dermatitis.

Article (c) teaches that substance P produces an itch sensation, and that substance P elicited scratching in Section 3.2, "Behavioral effects of substance P and histamine" on pages 230 to 231.

Accordingly, Applicants submit that, in view of the above-mentioned arguments and the evidence provided [Articles (a) - (c)] the specification provides enablement for the scope of claims 8 and 9. Withdrawal of the rejection is respectfully requested.

Lastly, claims 13-18 are newly added and depend directly or indirectly from Claim 1 and are directed to subject matter recited in claims 8 and 9. Thus, claims 13-18 are also enabled for at least the reasons mentioned above.

II. Response to Rejection under 35 U.S.C. § 112, Second Paragraph

Claims 1-12 are rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as their invention. Specifically, the Examiner argues that "uracil derivative" is indefinite because it may be interpreted as a residue derived from the uracil compound or a modification to the compound.

Applicants have amended the claims by deleting "derivative" and inserting --compound--. Accordingly, Applicants respectfully request withdrawal of the rejection in view of the Amendment.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned attorney at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

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Involvement of Leukotriene B_4 in Substance P-Induced Itch-Associated Response in Mice

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Intradermal injection of substance P elicits an itch sensation in human subjects and an itch-associated response in mice. The substance P-induced itch-associated response in mice is not inhibited by antihistamine. Therefore, the mechanisms of substance P-induced itch-associated response are unclear. In this study, we demonstrated one of the mechanisms. Substance P induces an arachidonate cascade to produce prostaglandins and leukotriene. In this study we considered whether arachidonate metabolites are involved in the substance P-induced itch-associated response. A phospholipase A_2 inhibitor arachidoryl-trifluoromethyl ketone inhibited the substance P-induced itch-associated response in mice. Pretreatment with the glucocorticoids betamethasone and dexamethasone also produced inhibition of the substance P-induced itch-associated response in mice as well as humans. The 5-lipoxygenase inhibitor zileuton, but not the cyclooxygenase inhibitors indomethacin and diclofenac, suppressed substance P-induced itch-associated response. The leukotriene B_4 receptor antagonist 5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5E-hexenyl]oxyphenoxy]valeric acid produced inhibition, whereas pranlukast (leukotriene $C_4/D_4/E_4$ receptor antagonist) and 5(Z)-7-[1S,2S,3S,5R-3-(trans-b-styren)sulfonamido-6,6-dimethylbicyclo(3,1,1)hept-2-yl]-5-heptenoic acid (EP_1 receptor antagonist) were without effect. Furthermore, when the production of leukotriene B_4 and prostaglandin E_2 was measured in skin injected with substance P and in mouse keratinocytes applied with substance P, the level of both products increased. As leukotriene B_4, but not prostaglandin E_2, also induces the itch-associated response in mice, these results suggest that leukotriene B_4 and keratinocytes, cutaneous cells which produced leukotriene B4, play an important role in substance P-induced itch-scratch response in mice. Leukotriene B_4 receptor antagonist and 5-lipoxygenase inhibitor may be novel antipruritic drugs. *Key words: keratinocyte/leukotriene B_4 receptor/5-lipoxygenase/tachykinin NK_1 receptor/WBB6F1 W/W^v. J Invest Dermatol 117:1621–1626, 2001*

Itch is a skin sensation that provokes a desire to scratch. This sensation accompanies various skin diseases (e.g., atopic dermatitis, contact dermatitis, and urticaria) and several systemic disorders (e.g., chronic renal failure and cholestasis). H_1 histamine receptor antagonists are the drugs of first choice for the treatment of itch, but many pruritic diseases except acute urticaria respond poorly to the H_1 receptor antagonists (Wahlgren, 1991). The precise mechanisms and mediators of itch in most pruritic diseases are unclear.

When applied to the skin, substance P (SP) elicits an itch sensation in human subjects (Hägermark *et al*, 1978; Barnes *et al*, 1986). This peptide is speculated to be involved in hemodialysis-associated pruritus (Kaku *et al*, 1990) and the pruritus of atopic dermatitis (Heyer *et al*, 1991) and psoriasis (Ellis *et al*, 1993). In mice, SP elicits scratching behavior (Kuraishi *et al*, 1995; Andoh *et al*, 1998). This response is suppressed by pretreatment with naloxone, capsaicin, and compound 48/80 (Andoh *et al*, 1998). Similarly, several kinds of human itching are alleviated by treatment with naloxone (Summerfield, 1980; Bernstein *et al*, 1982; Bergasa *et al*, 1995) and capsaicin (Tóth-Kása *et al*, 1986; Wallengren, 1991; Knight and Hayashi, 1994), and SP-induced itching is inhibited by pretreatment with compound 48/80 (Hägermark *et al*, 1978). These similarities suggest that SP-induced scratching behavior of the mouse is an itch-associated response. SP is generally believed to elicit itch through the release of histamine from mast cells. In human subjects, the H_1 histamine receptor antagonist chlorcyclizine inhibits an itch sensation induced by intradermal (i.d.) injection of low, but not high, doses of SP (Hägermark *et al*, 1978). Intradermal SP releases histamine from human skin for 20 min, a duration that is longer than the duration of itching (Barnes *et al*, 1986). In mice, an i.d. injection of SP elicits itch-associated response in normal mice and mast-cell-deficient mice as well (Andoh *et al*, 1998). Neurokinin-1 (NK_1) tachykinin receptors are involved in the itch-associated response induced by SP (Andoh *et al*, 1998), but not in the histamine release from mast

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Abbreviations: $AACOCF_3$, arachidoryltrifluoromethyl ketone; L-659 877, cyclo(Gln-Trp-Phe-Leu-Met); L-668 169, cyclo(Gln-D-Trp(NMe)Phe(R)Gly(ANC-2]Leu-Met)$_2$; LTB_4, leukotriene B_4; NK, neurokinin; ONO-4057, 5-[2-(2-carboxyethyl)-3-(6-(4-methoxyphenyl)-5E-hexenyl)oxyphenoxy]valeric acid; ONO-NT-012, 5(Z)-7-[1S,2S,3S,5R-3-(trans-b-styren)sulfonamido-6,6-dimethylbicyclo(3,1,1)hept-2-yl]-5-heptenoic acid; SP, substance P.

cells (Mousli et al, 1990). These findings taken together suggest that mast-cell-dependent and independent mechanisms are involved in SP-induced itch-associated response.

In human subjects, prostaglandin (PG) E_2 is a weak pruritogen (Hägermark and Strandberg, 1977) and prolongs experimentally induced itch (Hägermark and Strandberg, 1977; Fjellner and Hägermark, 1979). In addition, aspirin inhibits itching of patients with polycythemia vera (Fjellner and Hägermark, 1979). In animal experiments, although an i.d. injection of PGE_2 alone does not elicit itch-associated response (Andoh and Kuraishi, 1998), it enhances itch-associated response induced by serotonin (unpublished observation). On the other hand, an i.d. injection of leukotriene (LT) B_4 elicits apparent itch-associated response in mice (Andoh and Kuraishi, 1998), although it shows only weak effects at higher concentrations in mice (Andoh and Kuraishi, 1998) and humans (Camp et al, 1983). Thus, it seems interesting to examine whether the arachidonic acid cascade is involved in itch-associated response induced by SP in mice.

MATERIALS AND METHODS

Animals Unless otherwise mentioned, we used male ICR mice (Japan SLC, Shizuoka, Japan) of 5–6 wk of age. In some experiments, mast-cell-deficient mice (WBB6F1 W/W^v) were used at the age of 5 wk. They were housed under controlled temperature (23°C–25°C) and light (lights on from 08:00 to 20:00) conditions. Food and water were freely available. The experimental procedures for mice were conducted in accordance with the guidelines of the Guiding Principles for the Care and Use of Laboratory Animals approved by the Committee for Animal Experiments in Toyama Medical and Pharmaceutical University.

Drugs SP (Peptide Institute, Minoh, Japan) was dissolved in physiologic saline. Diclofenac sodium, arachidonyltrifluoromethyl ketone ($AACOCF_3$), cyclo(Gln-D-Trp(NMe)Phe(R)Gly[ANC-2]Leu-Met)$_2$ (L-668 169), and cyclo(Gln-Trp-Phe-Leu-Met) (L-659 877) were purchased from Research Biochemicals International (Natick, MA) and dissolved in physiologic saline. Dexamethasone, betamethasone, and indomethacin were purchased from Sigma Chemical (St. Louis, MO) and suspended in 0.5% sodium carboxymethylcellulose solution. Zileuton, 5-[2-(2-carboxyethyl)-3-[6-(4-methoxyphenyl)-5E-hexenyl]oxyphenoxy]valeric acid (ONO-4057), and pranlukast (all from Ono Pharmaceutical, Osaka, Japan) were suspended in 0.5% sodium carboxymethylcellulose solution. 5(Z)-7-[1S,2S,3S,5R-3-(trans-b-styren)sulfonamide-6,6-dimethylbicyclo(3,1,1)hept-2-yl]-5-heptenoic acid (ONO-NT-012) (Ono Pharmaceutical) was dissolved in equimoles of 1 N NaOH and then diluted with tap water. The sodium salt of LTB_4 (Ono Pharmaceutical) was dissolved in physiologic saline. LTD_4 was purchased from Sigma (St. Louis) and dissolved in ethanol and then diluted with physiologic saline. Fluo-3/AM and F-127 were purchased from Dojindo (Kumamoto, Japan).

Behavioral experiments The hair was clipped over the rostral part of the murine back the day before the experiment. Before behavioral recording, the mice (four animals per observation) were put into an acrylic cage (26 × 18 × 30 cm) composed of four cells (13 × 9 × 30 cm) for at least 1 h for acclimation. Immediately after an i.d. injection, the animals were put back in the same cells and their behaviors were videotaped using an 8 mm video camera for 1 h with all experimenters kept out of the observation room. Playing back of the video served for counting scratching behavior. The mouse generally scratches by the hind paws several times for about 1 s and a series of these movements was counted as one bout of scratching (Kuraishi et al, 1995). SP, LTB_4, and LTD_4 were injected i.d. in a volume of 50 µl into the interscapular part of the back. $AACOCF_3$, L-668 169, and L-659 877 were injected i.d. together with SP. Zileuton, ONO-4057, pranlukast, dexamethasone, and betamethasone were administered perorally 1 h before SP injection. Indomethacin, sodium diclofenac, and ONO-NT-012 were administered perorally 30 min before SP injection.

Isolation of mouse keratinocytes The clipped, ethanol-sterilized skin was isolated from the mouse that had been decapitated and bled. After washing it with phosphate-buffered saline containing 100 U per ml penicillin and 100 µg per ml streptomycin (PBS-PS), we removed connective tissue from the isolated skin and cut the skin into pieces. The pieces were washed twice with PBS-PS containing 0.02% ethylenediamine tetraacetic acid (EDTA), and treated with 0.25% trypsin dissolved in PBS-PS at 4°C for 6 h. The epidermis was carefully peeled from the skin, and gently shaken in PBS-PS containing 0.02% EDTA at room temperature until most epidermis preparations floated up. The floating preparations were passed through a stainless steel mesh with 40 µm pores and the filtrate was centrifuged at 150 × g at 4°C for 5 min. Precipitated cells were washed with 10 ml of Joklik-modified minimum essential medium containing 100 U per ml penicillin and 100 µg per ml streptomycin (MEM-PS), suspended in MEM-PS containing 20 ng per ml epidermal growth factor, 10 µg per ml insulin, and 10 ng per ml bovine pituitary extract, and cultured at 37°C in air containing 5% CO_2 until use.

Enzyme immunoassay The treated region of the skin (2 cm in diameter) was isolated 3 min after injection and immediately put into ice-chilled ethanol containing 10 µM indomethacin and 10 µM zileuton. After homogenization and centrifugation (at 3000 rpm for 5 min) of the skin sample, 2 ml of supernatant was diluted with 20 ml of ice-chilled 0.1 M acetic acid, applied to Bond Elute C_2 column (Varian, MA), and eluted with 3 ml ethyl acetate. After evaporation of the eluant, the residue was suspended in 150 µl of 0.01 M sodium bicarbonate buffer (pH 10.0), sonicated for 5 min, and then diluted with enzyme immunoassay buffer (Cayman Chemical, Ann Arbor, MI) for the assays of LTB_4 and PGE_2.

Primary cultures of the mouse keratinocytes were diluted by MEM-PS to 1 × 10^4 cell per 400 µl well. Drugs were added to the culture medium in a volume of 50 µl and then SP was added immediately (vehicle, L-668 169 and L-659 877), or 30 min (indomethacin) or 1 h later (dexamethasone and zileuton) in a volume of 50 µl. Samples were taken from the supernatant 5 min after SP application and diluted with enzyme immunoassay buffer (Cayman Chemical) for the assay. The amount of LTB_4 and PGE_2 was determined using EIA kits (Cayman Chemical).

Statistical analysis All data are presented as mean and SEM. Statistical significance was analyzed using the one-way analysis of variance followed by Dunnett's multiple comparisons. $p < 0.05$ was considered significant.

RESULTS

Effects of phospholipase A_2 inhibitor and glucocorticoids on SP-induced itch-associated response An i.d. injection of SP (100 nmol per site) elicited scratching of the skin around the injected site by the hind paws in mice (Fig 1a); the total number of responses by 1 h after injection was 75.7 ± 14.4 (mean ± SEM, $n = 8$). When injected together with SP, the phospholipase A_2 inhibitor $AACOCF_3$ (Street et al, 1993) (10–100 nmol per site) suppressed SP-induced itch-associated response in a dose-dependent manner (Fig 1b). A 1 h pretreatment with dexamethasone and betamethasone at peroral doses of 0.3–3 mg per kg also suppressed the itch-scratch response induced by SP (Fig 1c, d).

Effects of 5-lipoxygenase inhibitor and leukotriene receptor antagonists on SP-induced itch-associated response We examined the involvement of the 5-lipoxygenase pathway in the itch-associated response induced by SP. The 5-lipoxygenase inhibitor zileuton (Carter et al, 1991) at peroral doses of 10–100 mg per kg produced a dose-dependent inhibition of the SP action (Fig 2a). In addition, ONO-4057, an LTB_4 receptor antagonist (Kishikawa et al, 1991), at peroral doses of 10–100 mg per kg also produced a dose-dependent inhibition of the SP action (Fig 2b). On the other hand, the same doses of pranlukast, an antagonist to cysteinyl LTs (Nakagawa et al, 1992), did not affect the SP action (Fig 2c). In addition, i.d. injection of LTD_4 did not increase itch-associated response; the numbers of scratching following LTD_4 at doses of 0.01, 0.03, 0.1, and 0.3 nmol per site were 27.4 ± 3.5, 27.4 ± 5.5, 28.9 ± 8.5, and 14.4 ± 2.9 per h (mean ± SEM, $n = 8$ each), respectively.

Effects of cyclooxygenase inhibitors and prostaglandin receptor antagonist on SP-induced itch-associated response We examined the involvement of the cyclooxygenase pathway in the itch-associated response induced by SP. The cyclooxygenase inhibitors indomethacin (1–10 mg per

kg) and diclofenac sodium (3 and 10 mg per kg) did not inhibit the itch-associated response induced by SP, but instead there were increased tendencies (Fig 2d). In addition, ONO-NT-012, which blocks EP_1 PG receptors (Minami *et al*, 1995), did not affect the SP-induced itch-associated response (Fig 2e).

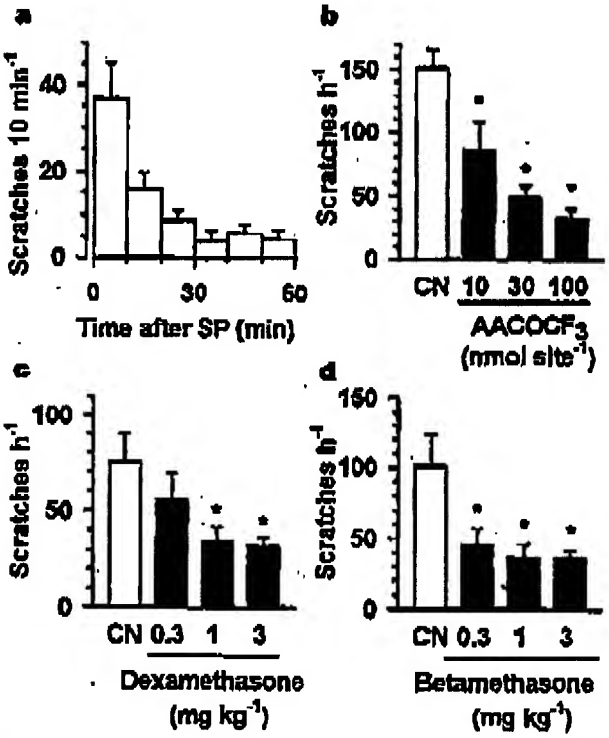

Figure 1. Inhibition of SP-induced itch-associated response by phospholipase A_2 inhibitor and glucocorticoids. (*a*) Time-course of itch-associated response of the mouse after an intradermal injection of SP (100 nmol per site). (*b*) The phospholipase A_2 inhibitor $AACOCF_3$ suppresses SP-induced itch-associated response. The inhibitor was injected intradermally together with SP (100 nmol per site). (*c, d*) Glucocorticoids suppress SP-induced itch-associated response. Dexamethasone (*c*) and betamethasone (*d*) were administered perorally 1 h before the SP injection. Results (mean ± SEM) are of eight animals. *$p < 0.05$ compared with control (CN).

Effect of LTB_4 receptor antagonist on SP-induced itch-associated response in mast-cell-deficient mice When injected intradermally, SP (100 nmol per site) elicited an apparent itch-associated response with comparable potency in mast-cell-deficient mice (WBB6F W/W^v) (Fig 3). A 1 h pretreatment with ONO-4057 at a peroral dose of 100 mg per kg markedly suppressed SP-induced itch-associated response in mast-cell-deficient mice and control mice (Fig 3).

Contents of LTB_4 and PGE_2 in the skin An i.d. injection of SP (100 nmol per site) produced a 2.9-fold increase in the cutaneous content of LTB_4, and this action was almost abolished by $AACOCF_3$ (100 nmol per site), dexamethasone (3 mg per kg), and zileuton (100 mg per kg) (Fig 4a); these dosages suppressed SP-induced itch-associated response (Figs 1b, c, 2a). The NK_1 receptor antagonist L-668 169 at 50 nmol per site, a dose that suppressed the SP-induced itch-associated response (Andoh *et al*, 1998), also abolished SP-induced increase in LTB_4 content, whereas indomethacin (10 mg per kg) was without effect (Fig 4a).

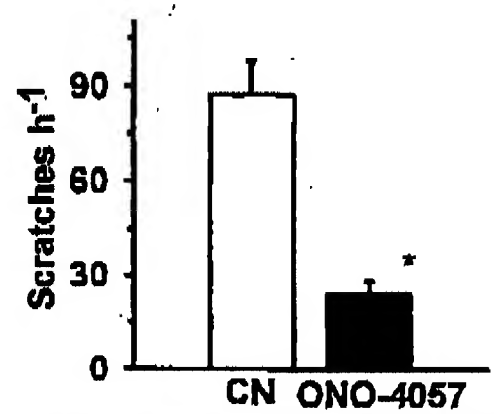

Figure 3. Inhibition of SP-induced itch-associated response by ONO-4057 in mast-cell-deficient mice. SP (100 nmol per site) was injected intradermally. ONO-4057 (100 mg per kg) was administered perorally 1 h before SP injection. Results (mean ± SEM) are of eight animals. *$p < 0.05$ compared with control (CN).

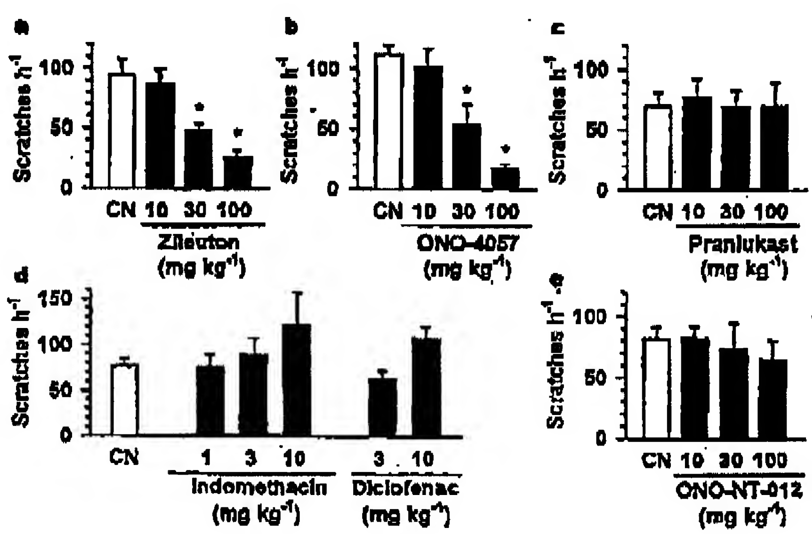

Figure 2. Effects of inhibition of eicosanoid systems on SP-induced itch-associated response. The mouse was given an i.d.injection of SP (100 nmol per site) and scratching behavior was counted for 1 h. (*a*) Zileuton, (*b*) ONO-4057, and (*c*) pranlukast were administered perorally 1 h before SP injection, and (*d*) indomethacin, (*d*) diclofenac, and (*e*) ONO-NT-012 were administered 30 min before. Results (mean ± SEM) are of eight animals. *$p < 0.05$ compared with control (CN).

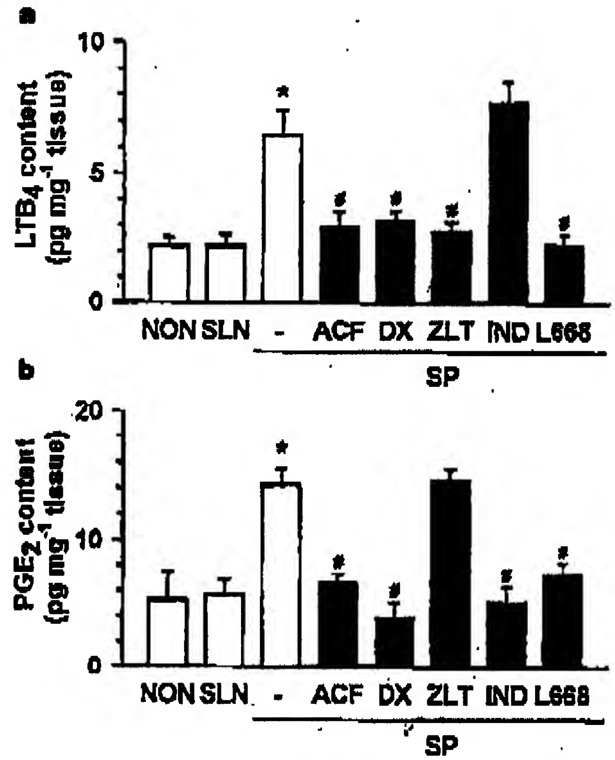

Figure 4. The production of LTB_4 and PGE_2 after SP injection in mouse skin. SP (100 nmol per site) and saline (SLN) were injected intradermally and 5 min later the contents of LTB_4 and PGE_2 in the treated skin were determined. $AACOCF_3$ (ACF; 100 nmol per site) and L-668 169 (L668; 50 nmol per site) were injected with SP. Dexamethasone (DX; 3 mg per kg) and zileuton (ZLT; 100 mg per kg) were administered perorally 1 h before SP injection, and indomethacin (IND; 10 mg per kg) was 30 min before. Results (mean ± SEM) are of six animals. *$p < 0.05$ vs SLN; #$p < 0.05$ vs SP alone. NON, nontreatment.

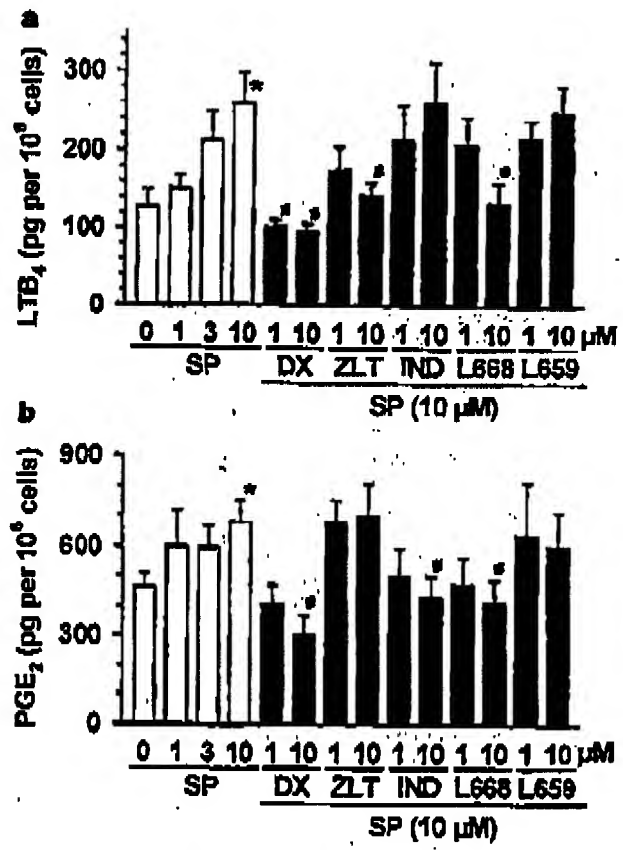

Figure 5. The SP-induced production of LTB_4 and PGE_2 in mouse keratinocytes. SP (10 µM) was applied to the primary culture of keratinocytes that had been isolated from mouse skin, and 5 min later the supernatant was taken for the assay. Dexamethasone (DX) and zileuton (ALT) were administered 1 h before SP, and indomethacin (IND) 30 min before. L-668 169 (L668), L-659 877 (L659), and vehicle (SP alone) was administered immediately before SP. Results (mean ± SEM) are of six experiments. *$p < 0.05$ vs without SP; #$p < 0.05$ vs 10 µM SP alone.

An i.d. injection of SP (100 nmol per site) produced a 2.5-fold increase in the cutaneous content of PGE_2 (Fig 4b). Although $AACOCF_3$ (100 nmol per site), dexamethasone (3 mg per kg), and L-668 169 (50 nmol per site) inhibited this action, zileuton (100 mg per kg) was without effect (Fig 4b). Indomethacin (10 mg per kg) abolished the SP-induced production of PGE_2 (Fig 4b).

Production of LTB_4 and PGE_2 in keratinocytes As mouse keratinocytes responded to SP in the above experiments, we examined whether SP would increase the production of LTB_4 and PGE_2 in the keratinocytes. A bath application of SP in concentrations of 1–10 µM to the primary cultures of mouse keratinocytes increased the production of LTB_4 in a concentration-dependent manner, with a 2-fold increase at a concentration of 10 µM (Fig 5a). Such SP effect was abolished by pretreatment with dexamethasone in concentrations of 1 and 10 µM. This effect was also inhibited by pretreatment with zileuton (1 and 10 µM) in a concentration-dependent manner, with complete inhibition at 10 µM, whereas indomethacin (1 and 10 µM) was without effect. L-668 169 (1 and 10 µM) exerted a concentration-dependent inhibition on the LTB_4 production induced by SP, whereas the NK_1 receptor antagonist L-659 877 (1 and 10 µM) was without effect.

SP (1 and 10 µM) also increased the production of PGE_2 in the mouse keratinocytes; the concentration of PGE_2 following 10 µM SP was 1.5 times that of untreated controls (Fig 5b). The SP action was inhibited by L-668 169 (1 and 10 µM), but not by L-659 877 (1 and 10 µM). Dexamethasone and indomethacin at concentrations of 1 and 10 µM inhibited PGE_2 production, whereas zileuton was without effect at the same concentrations (Fig 5b).

DISCUSSION

The phospholipase A_2 inhibitor $AACOCF_3$ at a dose that inhibited SP-induced production of PGE_2 and LTB_4 markedly inhibited SP-induced itch-associated response. The results show an important role of the arachidonic acid cascade in SP-induced itching. In this study, furthermore, we determined the role of 5-lipoxygenase and cyclooxygenase pathways in the SP action. The LTB_4 receptor antagonist ONO-4057 markedly suppressed the itch-associated response of the mouse to SP. In addition, SP markedly increased the content of LTB_4 in the skin at a dose that elicited itch-associated response. LTB_4 is a potent pruritogen; LTB_4 at a dose of 0.03 nmol per site elicits about a hundred scratch bouts per hour (Andoh and Kuraishi, 1998), which is comparable to the effect of SP at a dose of 100 nmol per site. Taken together, these findings strongly suggest that LTB_4 is involved in SP-induced itching in mice. Zileuton selectively inhibited SP-induced increase in the content of LTB_4, but not PGE_2, in the skin and markedly inhibited itch-associated response following SP injection, a finding suppor-

ing the view that LTB_4 plays an important role in SP-induced itching. In humans, LTB_4 is increased in the lesioned skin of patients with pruritic diseases such as atopic dermatitis and psoriasis (Brain *et al*, 1984; Ruzicka *et al*, 1986). Pruritus of chronic hemodialysis patients is alleviated by azelastin (Matsui *et al*, 1994; Kanai *et al*, 1995), an agent that inhibits LTB_4 receptor and LTB_4 production. These findings taken together suggest that the suppression of LTB_4 production is a mechanism of acute antipruritic action of glucocorticoids and provide the possibility that LTB_4 receptors and 5-lipoxygenase are novel targets for the development of antipruritic agents.

An i.d. injection of LTB_4 at doses of 0.001–0.03 nmol per site produces itch-associated response in a dose-dependent manner (Andoh and Kuraishi, 1998), whereas LTD_4 was without effect at doses of 0.01–0.3 nmol per site (present experiments). In addition, the cysteinyl LT antagonist pranlukast did not affect SP-induced itch-associated response. LTD_4 and LTC_4 are not pruritogenic after i.d. injection in human subjects (Camp *et al*, 1983). Therefore, cysteinyl LT may not be pruritogenic at least in the skin.

An i.d. injection of SP increased the content of PGE_2 in the skin. Although indomethacin (10 mg per kg) abolished the SP-induced increase in the cutaneous content of PGE_2, it did not inhibit the itch-associated response induced by SP. Similarly, diclofenac also did not suppress the SP-induced itch-associated response, and the EP_1 receptor antagonist ONO-NT-012 was without effect. In contrast, although zileuton markedly suppressed the SP-induced itch-associated response, it did not affect the cutaneous production of PGE_2. Therefore, PGE_2 may not play an important role in the pruritogenic action of SP. The results are consistent with the effects of aspirin-like agents on human itching; these agents generally do not suppress experimental and dermatitis-induced itching (Ståhle and Hägermark, 1985; Daly and Shuster, 1986). An i.d. injection of PGE_2, however, elicits a mild itching in human subjects (Hägermark and Strandberg, 1977; Fjellner and Hägermark, 1979). PGE_2 increases serotonin-induced itching in human subjects (Fjellner and Hägermark, 1979) and serotonin-induced itch-associated response in mice (unpublished observation). In addition, aspirin alleviates pruritus of patients with polycythemia vera (Fjellner and Hägermark, 1979). Therefore, we do not exclude the possibility of the involvement of PGE_2 in the itching of some pruritic diseases.

SP markedly increased the production of LTB_4 and slightly PGE_2, both of which were abolished by the NK_1 tachykinin receptor antagonist. SP also increases $[Ca^{2+}]_i$ in cultured mouse keratinocytes (Koizumi *et al*, 1994). The results indicate that SP acts directly on the NK_1 receptors of the mouse keratinocytes to produce eicosanoids, especially LTB_4. Considering that the keratinocytes are the largest cell group in the epidermis, the data suggest that the epidermal keratinocytes are the primary site of the LTB_4-producing action of i.d. SP. The NK_1 receptor antagonist L-668 169 abolished the production of LTB_4 in the skin and keratinocytes, whereas the NK_2 receptor antagonist L-659 877 was without effect. These results are consistent with behavioral experiments in which SP-induced itch-associated response was markedly suppressed by L-668 169 but not by L-659 877 (Andoh *et al*, 1998). Thus, the keratinocytes may play an important role in the production of a pruritogenic mediator LTB_4. The ability of human keratinocytes to produce LTB_4 is in dispute. Some workers reported on 5-lipoxygenase expression in human keratinocytes (Janssen-Timmen *et al*, 1995), whereas others have not found 5-lipoxygenase (Breton *et al*, 1996). Human keratinocytes have LTA_4 hydrolase activity, however (Ikai *et al*, 1994; Iversen *et al*, 1994), and transform exogenous and polymorphonuclear-cell-derived LTA_4 into LTB_4 (Iversen *et al*, 1994). Therefore, keratinocytes in human epidermis may produce LTB_4 under certain dermatitis conditions. Cutaneous mast cells may release LTB_4 (Harris *et al*, 1998). ONO-4057 significantly inhibited SP-induced itch-associated response in mast-cell-deficient mice, however. Therefore, the mast cells may not be the primary source of pruritogenic LTB_4 in the epidermis. Thus, keratinocytes rather than mast cells may be important target cells of antipruritic agents.

Itch signals may be mediated mainly by C fibers (Schmelz *et al*, 1997). LTB_4 sensitizes cutaneous C fiber nociceptors (Martin *et al*, 1988). In addition, in our preliminary experiments, LTB_4 increased the concentration of intracellular Ca^{2+} ions in primary cultured mouse dorsal root ganglion neurons, especially capsaicin-sensitive neurons (unpublished observations); most C fibers are sensitive to capsaicin (Holzer, 1991). Repeated treatment with capsaicin cream suppresses the itch of patients with pruritic diseases, such as chronic renal failure with hemodialysis treatment (Breneman *et al*, 1992), pruritic psoriasis (Ellis *et al*, 1993), notalgia paresthetica (Wallengren, 1991), and hydroxyethylstarch-induced pruritus (Szeimies *et al*, 1994). In addition, repeated treatment with capsaicin inhibits experimental itch induced by histamine in humans (Tóth-Kása *et al*, 1986) and itch-associated response induced by SP in mice (Andoh *et al*, 1998). Thus, it is suggested that itch is mediated by capsaicin-sensitive primary afferents. Therefore, LTB_4 produced by SP-stimulated keratinocytes (and the other cutaneous cells) might act directly on primary afferent terminals to produce itch signals.

Glucocorticoids suppressed SP-induced itch-associated response. Considering that glucocorticoids suppress the production of arachidonic acid metabolites by inhibiting phospholipase A_2 (Piltch *et al*, 1989), these results raised the possibility that the inhibitory action of glucocorticoids on the SP action was substantially due to the inhibition of the arachidonic acid cascade. Recently, however, it has been reported that glucocorticoids regulate the expression of cyclooxygenase-2, but not cyclooxygenase-1 (Zhang *et al*, 1999). In this study, treatment with glucocorticoids 1 h before the SP application inhibited the production of LTB_4 and PGE_2 in murine skin and keratinocytes. Although the detail of the mechanisms is unknown from this study, these results suggest that the acute action(s) of glucocorticoids may include the inhibition of enzyme activities including phospholipase A_2. Furthermore, we examined whether dexamethasone has the antagonistic activity of LTB_4 receptors. Dexamethasone (3 mg per kg) markedly suppressed both itch-associated response and LTB_4 production induced by SP, whereas it did not affect the LTB_4-induced itch-associated response (unpublished observation). Therefore, the inhibition of the production, rather than the action, of LTB_4 may be responsible for the suppressive action of dexamethasone on itch-associated response. Thus, the inhibition of the production of LTB_4 may be involved in the inhibitory effect of glucocorticoids on SP-induced itch-associated response.

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Increased number of immunoreactive nerve fibers in atopic dermatitis

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The presence of immunologic markers for neurofilaments, neuropeptides of sensory nerve fibers (Calcitonin gene-related peptide and substance P), for noradrenergic innervation (neuropeptide Y and Tyrosine hydroxylase), and Neuron-specific protein 9.5 was evaluated in frozen tissue sections from normal skin (n = 34) and from skin biopsies manifesting urticaria (n = 6), leukocytoclastic vasculitis (n = 4), systemic lupus erythematosus (n = 23), and atopic dermatitis (n = 40, of which 16 were from lesions induced by epicutaneous atopic allergen patch tests). In some normal skin specimens immunoreactive nerve fibers expressing Neuron-specific protein 9.5 were observed in the epidermis, dermis, and around blood vessels. For the other markers, immunolabeling was mainly observed in the dermis around blood vessels. Neurofilaments, which are scarce in normal skin epidermis, were present in higher density in the epidermis of affected skin in all disease conditions. Biopsies from urticaria and systemic lupus erythematosus showed a decrease in density of fibers immunolabeled for neuropeptides substance P and Calcitonin gene-related peptide and for Neuropeptide Y. In biopsies from skin with atopic dermatitis, an increased density of fibers was observed for all markers except Neuropeptide Y and Tyrosine hydroxylase. In this group, biopsies from positive atopic allergen patch tests showed an enhanced density of fibers labeled by antibody to Neuron-specific protein 9.5 and a lower density in labeling for Tyrosine hydroxylase. The data indicate a potential role of innervation and neuropeptides in dermatoses like atopic dermatitis. (J ALLERGY CLIN IMMUNOL 1992;90:613-22.)

Key words: Atopic dermatitis, dermatoses, inflammation, innervation, neuropeptides, skin

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The human skin is equipped with a rich supply of nerve elements serving receptor as well as effector functions. Environmental stimuli activate afferent sensory neurons that, by intervention of the central nervous system, give rise to mechanoreceptive (touch), thermal, and pain sensitization. Effector activities, for example, vasoconstriction/dilation and sweating, are mediated by impulses originating in the central nervous system and by the axon reflex of sensory nerves in the periphery. Varicosities along these nerves release neuropeptides that can act on specific receptors. These peptides are necessary for the initiation of inflammatory and reparative processes in injured skin.[1-6]

Altered patterns of cutaneous innervation have been reported in many inflammatory dermatoses, including psoriasis, leprosy, and eczema.[7-11] Similar observations have been made for urticaria on evidence from tissue-fluid analysis and capsaicin-induced prevention of urticarial responses.[12, 13] A symptom of these skin disorders is pruritus, where sensory C and possibly A Δ fibers are stimulated in the superficial dermis.[6, 10] Peptidergic mediators have been implicated in skin inflammatory disorders.[11] Immunocytochemical and radioimmunoassay studies have shown elevated levels of substance P (SP), Calcitonin gene-related peptide (CGRP), Neuropeptide Y (NPY), Vasoactive intestinal peptide, and somatostatin in blister tissue and fluids.[11, 13] These neuropeptides can manifest immunomodulatory activity,[14] and they contribute to the cross talk between the autonomic and sensory nervous systems and the immune system in the skin.[15]

In a retrospective analysis we investigated the presence of neurofilaments, CGRP, SP, NPY, Tyrosine hydroxylase (TH), and Neuron-specific protein 9.5 (PGP9.5). This analysis was performed by analysis of immunohistochemistry on skin biopsies from patients with urticaria, vasculitis, systemic lupus erythematosus (SLE), and atopic dermatitis (AD).

Abbreviations used

SP:	Substance P
CGRP:	Calcitonin gene-related peptide
NPY:	Neuropeptide Y
TH:	Tyrosine hydroxylase
PGP9.5:	Neuron-specific protein 9.5
SLE:	Systemic lupus erythematosus
AD:	Atopic dermatitis
PBS:	Phosphate-buffered saline

MATERIAL AND METHODS

Biopsies

This retrospective study included 40 biopsies from patients with AD (21 males and 19 females; age range, 1.5 to 72 years; median age, 20 years). Twenty-four of these biopsies were from affected skin and showed the histologic characteristics of AD. Sixteen biopsies were taken from lesions after stripping the epidermis, and subsequent epicutaneous patch testing was performed with house dust, house dust mite, and grass pollen allergens according to methods previously described.[16] Twenty-three biopsies were from patients with SLE (14 females, 9 males; age range, 0 to 72 years; median age, 33 years). Histopathologic study of this material showed changes characteristic of SLE, and immunofluorescence demonstrated granular deposits of immunoglobulins and complement along the epidermal basement membrane. Four biopsies from patients with vasculitis (3 females, 1 male; age, 11 to 36 years; median, 30 years) were analyzed. Histopathologic study of that material revealed leukocytoclastic vasculitis; with immunofluorescence, immunoglobulin and complement deposits were observed in superficial blood vessel walls. Six biopsies were from patients with urticaria (4 females, 2 males; age range, 24 to 69 years; median, 46 years). With histopathologic study some characteristics of urticaria were manifest. Thirty-four skin biopsies showing no histologic abnormalities served as controls; 31 samples were biopsies and 3 samples were obtained at autopsy (20 males, 14 females; age range, 15 to 79 years; median, 37 years). Some of these specimens were from uninvolved skin of patients in the disease categories mentioned above. Except for autopsy tissue, almost all samples were 3 mm punch biopsies. Biopsies from patients were from the affected skin; those from controls were mainly taken from the extensor site of the upper arm. In all cases the histopathologic diagnosis was made on formalin-fixed tissue embedded in paraffin. The samples analyzed in this study were snap frozen and stored at −70° C before analysis.

Immunohistochemistry

Frozen sections of 10 μm thickness were taken up in series of three consecutive sections onto glass slides coated with gelatin. The slides were fixed in 100% acetone for 10 minutes at room temperature. The primary antisera are listed in Table I. An avidin-biotin immunoperoxidase method was used. First, sections were preabsorbed with 10% normal goat (or horse) serum in phosphate-buffered saline (PBS) for 15 minutes. Then the sections were incubated for 1 hour with primary antiserum at the predetermined optimal dilution (Table I). They were subsequently rinsed in PBS and incubated for 30 minutes with biotinylated goat anti-rabbit (1:250) (or horse anti-mouse [1:150]) serum (Vector Laboratories, Burlingame, Calif.). Antisera were diluted in PBS supplemented with 1% normal goat (or horse) serum. The sections were rinsed in PBS and finally incubated for 30 minutes with an avidin-biotin complex (Vector Laboratories) in PBS supplemented with 1% normal goat (or horse) serum. After rinsing, the immunoreaction was visualized by use of a mixture of 3'3'-diaminobenzidine tetrahydrochloride (0.35

TABLE I. Antibodies applied in this study

Antigen	Species	Origin*	Dilution	Reciprocal Remarks
Neurofilament 200kd	Mouse	Sigma†	15,000	Neuron-specific cytoskeletal protein
Neurofilament 160kd	Mouse	Sigma†	15,000	Neuron-specific cytoskeletal protein
Neurofilament 68kd	Mouse	Sigma†	700	Neuron-specific cytoskeletal protein
CGRP	Rabbit	Amersham‡	150	Neuropeptide, sensory nerves
SP	Rabbit	Amersham§	200	Neuropeptide, sensory nerves
NPY	Rabbit	Amersham‖	30	Neuropeptide, noradrenergic innervation
TH	Mouse	Ultraclone¶	100	Enzyme involved in noradrenalin synthesis
PGP9.5	Rabbit	Ultraclone#	2,000	Neural-neuroendocrine-specific carboxyl-terminal hydrolase

*Origin: Amersham, Bucks, UK; Sigma Chemical Co., St. Louis, Mo.; Ultraclone, Incstar, Stillwater, Minn.
†Specification by the supplier: Raised to neurofilaments purified from pig spinal cord; IgG1 subclass; no crossreactivity to other intermediate filament proteins; reacts to neurofilaments of 200kd, 160 kd, or 68 kd.
‡Specification by the supplier: raised to rat CGRP conjugated to bovine serum albumin; crossreactivity, none detected with other peptides.
§Specification by the supplier: raised to synthetic SP conjugated to bovine serum albumin; crossreactivity, none detected with amphibian bombesin.
‖Specification by the supplier: raised to unconjugated synthetic porcine NPY: crossreactivity, no significant cross-reactivity with PYY.
¶Specification by the supplier: raised to TH from rat PC12 cells; IgG1 subclass: recognizes an epitope in the midportion of the TH molecule where extensive species homology exists; no crossreactivity to dihydropterine reductase, dopamine-β-hydroxylase, phenylethanolamine-N-methyltransferase, phenylalanine hydroxylase, and tryptophan hydroxylase (western blotting).
#Specification by the supplier: raised to human PGP9.5.

gm/L), nickel-ammoniumsulfate (6-hydrate, 25 gm/L) and H_2O_2 (0.1%) for 3 to 5 minutes. Sections were counterstained with Nuclear Fast Red (Sigma Chemical Co., St. Louis, Mo.), dehydrated, and embedded in synthetic mounting medium DPX (Aldrich Chemical Co., Milwaukee, Wis.). The immunolabeling product for individual antibodies is designated as "immunoreactive." Control material was incubated with an irrelevant antiserum, including a rabbit antimouse antiserum (Dakopatts; DAKO Immunoglobulins, Glostrup, Denmark) or a monoclonal antibody to human IgD (Dakopatts) of the same subclass (IgG1) as the monoclonal antibodies applied. The control procedure also included the replacement of primary antisera with PBS. No staining was observed, with the exception of eosinophilic and neutrophilic polymorphonuclear granulocytes, when present.

Semiquantitative scoring

The density of immunoreactive fiberlike structures in three consecutive sections was assessed by two independent observers. In cases of disagreement, mean scores were taken. The scores were expressed as absent (0), incidental (0.5), few (1), moderate (2), many (3), and abundant (4). Scores were given in relation to each of the following skin components: epidermis, superficial dermis, and superficial blood vessels. Also the occurrence in hair follicles or in sebaceous and eccrine sweat glands, if present, was noted but not scored. Statistical analysis was performed for groups defined according to the histopathologic diagnoses mentioned herein, by means of the nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) and the Mann-Whitney U test. The software applied was SPSS.PC+ version 3.0. For individual groups of biopsies, the difference in score with regard to normal skin was calculated. In addition, for the AD group the difference between affected skin and skin after patch testing was analyzed. Differences were considered significant at values of $p < 0.05$.

RESULTS

The semiquantitative scores of individual markers in epidermis, dermis, and around blood vessels, for individual disease categories and controls, are shown in Table II. Scores with the most significant differences after aggregation are graphically represented in Fig. 1. In the following section we describe the immunohistology and statistical results for individual markers.

Neurofilaments

The distribution and density of immunoreactive fibers observed was similar for labeling by antibodies to neurofilament 200, neurofilament 160, and neurofilament 68. The aggregated data are presented in Fig. 1, *a* in combined form for the epidermis. In normal skin, some immunoreactive fibers were pres-

TABLE II A. Semiquantitative score of immunoreactive fibers in various skin compartments

		Neurofilament 200			Neurofilament 160			Neurofilament 68		
Category	No.	Epidermis	Dermis	Vessels	Epidermis	Dermis	Vessels	Epidermis	Dermis	Vessels
Normal control	34	0 0 - 0.5	1 0 - 3	1 0 - 2	0 0 - 1	0 0 - 1	1 0.5 - 2	0 0 - 1	2 0 - 3	1 1 - 3
Vasculitis	4	1 0 - 2	1 1 - 2	0 0 - 2	1 0 - 1	1 0 - 2	1 0 - 1	0 0 - 2	1 0 - 2	0 0 - 2
SLE	23	0.5 0 - 2	1 0 - 3	1 0 - 2	0 0 - 0.5	1 0.5 - 3	1 1 - 2	0.5 0 - 0.5	2 0.5 - 3	1 1 - 3
Urticaria	6	1 0 - 2	1 1 - 3	1 1 - 2	0 0 - 1	1 0 - 3	2 0.5 - 3	1 0 - 2	2 1 - 2	2 1 - 3
Atopic dermatitis affected skin	24	0.5 0 - 1	2 1 - 4	2 0 - 3	0 0 - 1	1 0.5 - 3	1 0 - 3	1 0 - 2	2 0.5 - 3	2 0 - 3
Atopic dermatitis patch test	16	1 0 - 2	2 0.5 - 3	1 0.5 - 2	1 0 - 2	1 1 - 3	2 1 - 2	1 0 - 2	2 1 - 3	2 1 - 3

TABLE II B. Semiquantitative score of immunoreactive fibers in various skin components

		CGRP			SP			NPY		
Category	No.	Epidermis	Dermis	Vessels	Epidermis	Dermis	Vessels	Epidermis	Dermis	Vessels
Normal control	34	0 0 - 1	0 0 - 2	0 0 - 2	0 0 - 0.5	0.5 0 - 1	0.5 0 - 1	0 0 - 1	0.5 0 - 2	1 0 - 2
Vasculitis	4	0 0 - 0	0 0 - 0	0 0 - 1	0 0 - 0	0 0 - 1	0 0 - 0.5	0 0 - 0	0 0 - 2	0.5 0 - 1
SLE	23	0 0 - 0.5	0 0 - 2	0 0 - 1	0 0 - 0	0 0 - 0.5	0.5 0 - 0.5	0 0 - 0	0 0 - 1	0 0 - 1
Urticaria	6	0 0 - 0	0 0 - 2	0 0 - 0	0 0 - 0	0 0 - 0	0 0 - 0.5	0 0 - 0	0 0 - 0	0 0 - 1
Atopic dermatitis Affected skin	24	0 0 - 0	1 0 - 1	1 0 - 1	0 0 - 0	1 0 - 1	0.5 0.5 - 1	0 0 - 1	0 0 - 2	0 0 - 2
Atopic dermatitis Patch test	16	0 0 - 0	1 0.5 - 1	1 0 - 1	0 0 - 0	1 0.5 - 2	1 0.5 - 2	0 0 - 0.5	1 0 - 2	0 0 - 1

Data presented are median values and ranges determined by semiquantitative scoring.

ent in the superficial dermis and around blood vessels. Only rarely was labeling in the epidermis observed (Fig. 2, *a*). The presence of immunoreactive fibers in vasculitis cases was similar to that seen in normal skin. A significantly higher density of immunoreactive neurofilament fibers was observed in the epidermis of lesional skin of patients with SLE, urticaria, and AD (Fig. 1, *a*, illustrated in Fig. 2, *b* and *c*). In the AD group, biopsies from affected skin did not differ from those taken from skin after patch tests. In the superficial dermis and around blood vessels, SLE biopsies manifested a higher density than normal skin

PGP9.5		
Epidermis	Dermis	Vessels
0 0 - 2	1 0 - 4	1 1 - 4
0 0 - 0	0.5 0 - 1	0 0 - 0
0 0 - 0.5	1 0 - 3	0 0 - 1
0 0 - 0.5	1 0 - 3	0.5 0 - 3
0 0 - 1	1 0 - 2	1 0 - 2
0 0 - 1	2 0 - 3	1 0 - 3

TH		
Epidermis	Dermis	Vessels
0 0 - 1	0 0 - 3	0.5 0 - 2
0 0 - 0	0 0 - 0	0 0 - 2
0 0 - 1	0 0 - 2	0 0 - 2
0 0 - 0	0.5 0 - 1	0.5 0 - 2
0 0 - 0	1 0 - 2	2 0 - 3
0 0 - 0	0 0 - 2	0 0 - 2

(p = 0.004) (Fig. 2, *c*). But SLE lesional skin with a marked mononuclear cell infiltrate contained very few immunoreactive-neurofilament fibers (Fig. 2, *d*).

Neuropeptides in sensory nerves (CGRP [Fig. 1, *b*] and SP)

In normal skin the distribution and relative density of immunoreactive CGRP and immunoreactive SP fibers were similar. They were very rarely seen in the epidermis, whereas they were more common around blood vessels and in association with trespassing nerve bundles. Similar observations were made in vasculitis biopsies. A lower mean density of immunoreactive CGRP was found in biopsies from patients with SLE, and a significantly higher density was observed in biopsies from patients with AD (Fig. 1, *b*, illustrated in Fig. 3). In skin with urticaria, a low mean density was observed, but in some cases clear labeling was seen in the upper dermis around blood vessels (Fig. 4).

Noradrenergic innervation (NPY [Fig. 1, *c*] and TH [Fig. 1, *d*])

In normal skin, immunoreactive NPY and immunoreactive TH fibers were observed around blood vessels and to a lesser extent around hair follicles and sebaceous glands. Except for a few cases, no intraepidermal staining was seen. Skin biopsies from patients with vasculitis showed similar results. The density of immunoreactive NPY fibers was lower in skin of patients with SLE and urticaria and similar to control specimens in skin of patients with AD (Fig. 2, *c*). A different picture was observed for TH. Here, the mean density of immunoreactive TH fibers was similar in skin of patients with SLE and urticaria to that found in normal skin, and a higher mean density was observed in skin of patients with AD (Fig. 2, *d*, illustrated in Fig. 5). Also, when compared with skin after patch testing, the higher mean density in affected skin was statistically significant (p = 0.005). Immunoreactive TH fibers were most dense around sweat glands, hair follicles, and blood vessels. Often leukocytes with a mast-cell appearance were labeled.

Other neural markers (PGP9.5 [Fig. 1, *e*])

Immunoreactivity for PGP9.5 in normal healthy skin was present in the epidermis, dermis, and around vessels. The highest mean density was observed in skin of patients with AD after patch test provocation, being statistically significant when compared with normal skin (p = 0.003) and affected skin of patients with AD (p = 0.03). In skin of patients with vasculitis, SLE, and urticaria, the mean density was similar to that found in normal skin.

DISCUSSION

The distribution and relative density of fibers that we found to be immunoreactive for neuropeptides and neurofilaments in normal healthy skin correspond to findings from the literature.[1-4] The highest density of immunoreactive fibers occurs in the dermis around blood vessels (Table II). This was observed for all

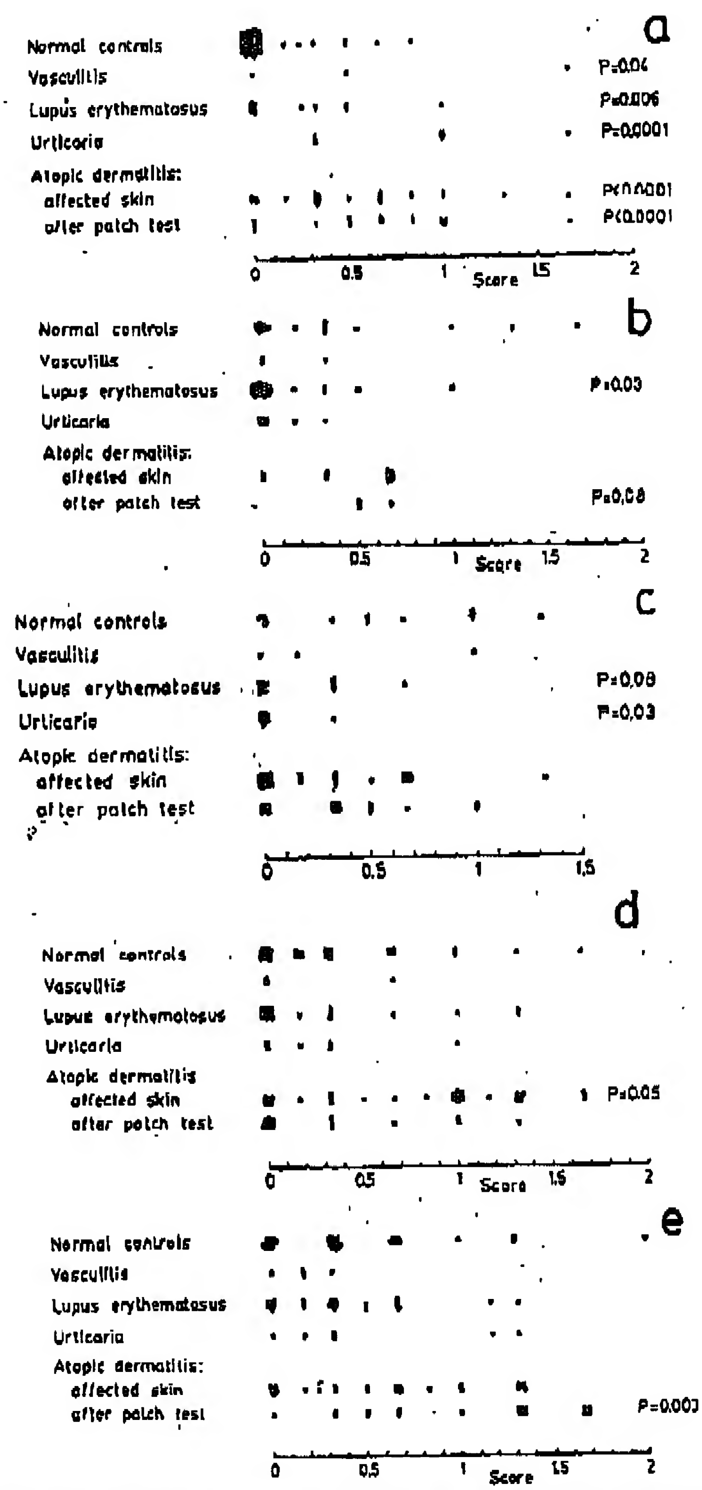

FIG. 1. Scores of individual neural markers in skin compartments. Figures present data of normal control biopsies of skin without histopathologic abnormalities and biopsies of skin with leukocytoclastic vasculitis, SLE, urticaria, and AD. Data subdivided into biopsies from affected skin and biopsies from lesions after epicutaneous patch tests with atopic allergens. Data shown: a, neurofilaments in the epidermis; b, mean of CGRP in epidermis, dermis, and around blood vessels; c, mean of NPY in epidermis, dermis, and around blood vessels; d, mean of TH in epidermis, dermis, and around blood vessels; e, mean of PGP9.5 in epidermis, dermis, and around blood vessels. Statistically significant differences from normal skin (Mann-Whitney U test) are indicated.

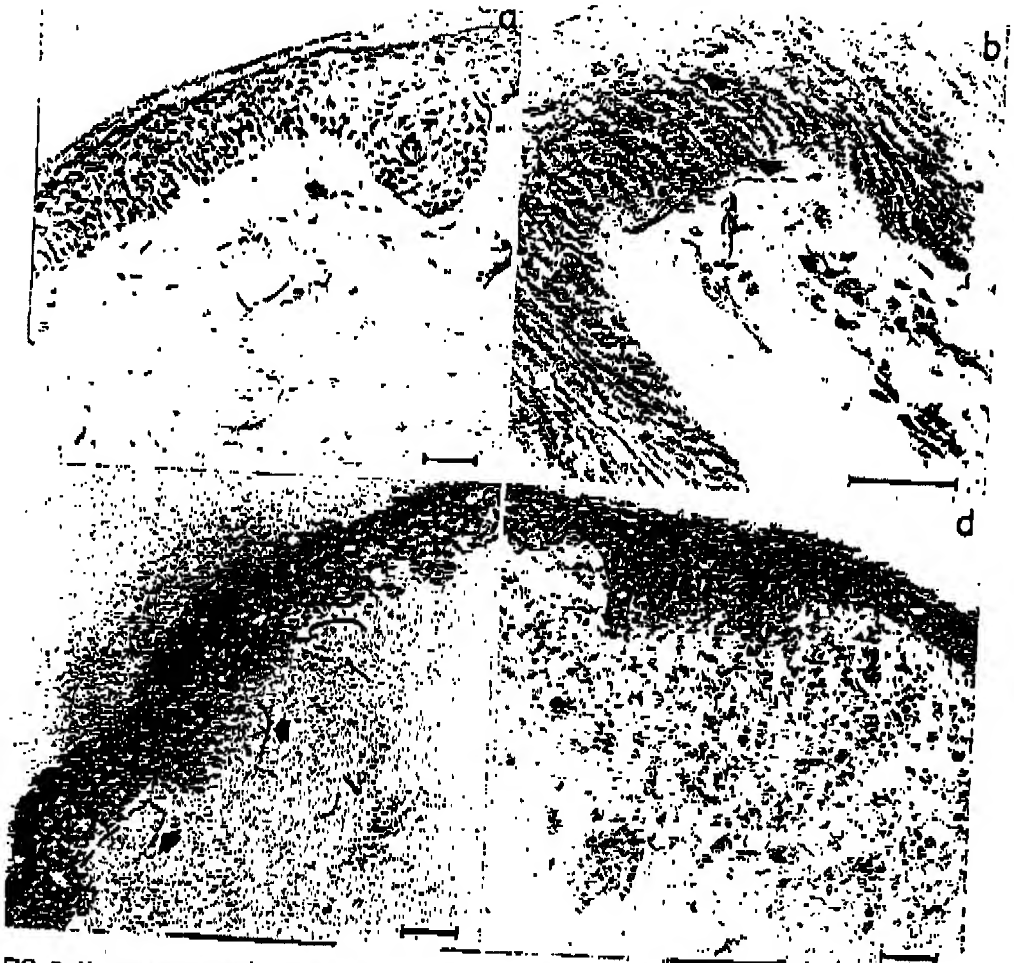

FIG. 2. Neurofilament immunolabeling. a, Normal healthy skin, without neurofilament 200 labeling product (score 0). b, Skin in patient with AD shows intraepidermal and subepidermal sensory nerves indicated by *arrows* (score 3). c, Skin in patient with SLE, shows many sensory nerves in and underneath the epidermis indicated by *arrows* (score 3). d, SLE lesional skin, with apparent absence of sensory nerve fibers (score 0). *Bar*, 50 μm.

markers investigated: neurofilaments, neuropeptides SP and CGRP associated with sensory nerves, NPY and TH as markers for noradrenergic innervation, and PGP9.5. For SP, the findings in the literature vary from negative to positive for fibers in the superficial dermis[3, 17]; our data confirm findings in the latter report. The immunoreactivity at other skin sites varied according to the antigen investigated. For instance, normal epidermis was negative for markers of noradrenergic innervation, and it was only rarely positive for PGP9.5, neurofilaments, and neuropeptides CGRP and SP. Similar observations have been reported by others.[1, 2, 4, 6] For visualization of cutaneous innervation, the choice of anti-PGP9.5 antibody or antibodies to neurofilaments is recommended.[3, 4]

The dermatoses investigated manifested a number of alterations in the immunoreactivity for nerve markers. This observation should be interpreted in light of the restrictions imposed by the study design. Since this investigation was retrospective, we were unable to perform a follow-up analysis on material from the same patient. Skin in patients with SLE, urticaria, and AD showed a statistically significant increase in the density of immunoreactive NF fibers in the epidermis (Fig. 1, *a*). Peptides associated with sensory nerves were decreased in skin of patients with SLE and urticaria (Fig. 1, *b*), and the scores for immunoreactive noradrenergic nerves (Fig. 1, *c*) were lower. On the other hand, skin of patients with AD showed a significant increase for all markers, except TH and NPY. For SP, a similar increase has been reported by others.[17] For NPY, Pincelli et al.[17] have shown staining of dendritic epidermal cells in skin of patients with AD. We were unable to reproduce these findings in

FIG. 3. Substance P immunolabeling in skin of a patient with AD. Immunoreactive fibers are associated with superficial dermal vessels (score 2), one of which is indicated by an *arrow*. Leukocytes also show labeling *(arrowhead)*. *Bar*, 25 μm.

FIG. 4. CGRP immunolabeling in urticarial skin. Immunoreactive fibers (one indicated by an *arrow*) are present in the superficial dermis and around dilated blood vessels (score 2). *Bar*, 25 μm.

the present study. This discrepancy may be ascribed to differences in study material, technique, and antibody applied. We conclude that the skin of patients with AD shows an increased density of immunoreactive nerve fibers. This does not occur in skin of patients with urticaria and SLE, except for immunoreactive neurofilament fibers in the epidermis.

A number of explanations for the observed changes in immunoreactive nerve fibers may be offered. First, the changes are caused by the disease process. This may underlie the lower density of immunoreactive profiles in the dermis of patients with SLE and urticaria; that is, the infiltrates may damage the nerve structures. For neurofilament immunolabeling, biopsies of skin of patients with SLE showed a higher expression in the dermis than controls, but this difference was not apparent when a mononuclear cell infiltrate was pronounced (Figs. 2, *c* and *d*). Second, the changes in nerve fibers may play a role in the pathogenesis of the inflammatory response. The enhanced expression of neurofilaments in the epidermis suggests such an explanation because nerves identified by neurofilament immunolabeling (Fig. 1, *a*) can serve as mediators of inflammation.[18] In patients with SLE and urticaria we did not find an increase in immunoreactivity for neuropeptides of sensory nerves, for example, SP and CGRP. For patients with AD a different picture emerges. The higher immunoreactivity for most of the markers investigated suggests that nerve components play a role in the disease process. Neuropeptides, including SP and CGRP,[19, 20] are able to produce inflammatory reactions on intradermal injection in normal skin. Also the weal and flare response after trauma is ascribed to sensory neuropeptides.[21] One mechanism triggering this response may operate via T lymphocytes, which are susceptible to stimulation by these peptides and subsequently initiate an inflammatory response.[22, 23] Patients with AD have been reported to be anergic or hyperreactive on such a challenge.[21, 22] This apparent discrepancy may be related to the ill-defined balance between the increased vulnerability to neuropeptide action on the one hand and exhaustion of the responding cellular components on the other. Alternatively, allergen challenge may

influence the neuropeptide status. In this study, biopsies taken from patients with AD after atopic allergen challenge showed a higher density for PGP9.5 and a lower density for TH than biopsies from affected skin. That difference forms a first indication of changes in nerve components after allergen challenge. This point warrants further study in serial biopsies from the same patients before a definite conclusion can be made.

In selecting the material for this retrospective study, our choice focused on inflammatory skin lesions. We conclude that lesions in patients with AD show detectable alterations in immunoreactive nerve profiles. This is also the case for neurofilament labeling in patients with urticaria, leukocytoclastic vasculitis, and SLE. In view of the present immunohistochemical data, future studies should focus on the role of sensory nerves and neuropeptides associated with sensory innervation in the etiology and pathogenesis of these dermatoses.

The authors thank Prof. Dr. Rudy E. Ballieux for his stimulating discussions of the design of this study and the analysis of the findings.

FIG. 6. TH immunolabeling in skin of a patient with AD. Immunoreactive TH fibers, indicated by an *arrow*, are associated with a superficial dermal blood vessel and perivascular infiltrate (score 3). *Bar*, 25 μm.

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Fish hypersensitivity. II: Clinical relevance of altered fish allergenicity caused by various preparation methods

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In double-blind, placebo-controlled, oral food challenges with fish, a 12-fold higher false-negative rate was found compared with other food antigens. In an effort to elucidate this discrepancy, cooked lyophilized fish extracts (used in double-blind, placebo-controlled, oral food challenges) were compared with cooked, nonlyophilized fish extracts (used in open challenges) by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunoblot, and ELISA-inhibition assays. Altered fish allergenicity as a result of food processing was examined with canned tuna and salmon. Forty-five children and young adults with food allergies, including 18 patients with IgE-mediated hypersensitivity to fish, were challenged with canned tuna. All 45 challenges with canned tuna were negative. Two of these patients are allergic to salmon and also have negative reactions to challenges with canned salmon. In vitro investigation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of tuna and salmon extracts revealed a striking loss of definable protein fractions in the canned fish extract when compared with raw and cooked fish extracts, and immunoblot analyses demonstrated minimal IgE-specific binding to the canned fish extracts. In addition, decreased allergenicity of the canned tuna and salmon was demonstrated by ELISA-inhibition assay and by negative oral challenges with canned salmon in two patients allergic to salmon. Collectively, these findings suggest that some of the major allergens responsible for IgE-mediated food allergy to fish are more labile than previously recognized. (J ALLERGY CLIN IMMUNOL 1992;90:622-9.)

Key words: Fish allergy, food hypersensitivity, double-blind, placebo-controlled oral food challenges, lyophilization, ELISA—inhibition assay, immunoblotting

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Allergenic food proteins are characteristically heat and acid stable, largely resistant to proteolysis and digestion, and their primary amino acid structures are bound by IgE.[1] Indeed, these inherent physicochemical characteristics of allergenic food proteins may play a major role in their allergenicity. Furthermore, despite the wide variety of foods consumed, only a small number are responsible for more than 90% of the food-allergic reactions in both pediatric and adult populations.[2]

Unfortunately for the population with food allergies, most allergenic food proteins require extensive alteration to decrease their allergenicity.[3-6] This is an issue that has gained notoriety in the infant formula

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Scratching behavior induced by pruritogenic but not algesiogenic agents in mice

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Abstract

We compared the behavioral effects of treatment with pruritogenic and algesiogenic agents in mice. The animals were given subcutaneous injections of pruritogenic agents, compound 48/80 (3–100 μg), substance P (10–300 μg) and histamine (3–300 μg), and algesiogenic agents, capsaicin (30 and 100 μg) and dilute formalin (5 mg of formaldehyde), into the rostral back, and scratching of the injected site by the hind paws was counted. Compound 48/80 and substance P dose dependently elicited the scratching behavior, but histamine, capsaicin and dilute formalin were without significant effects at the doses examined. These results suggest that compound 48/80- and substance P-induced scratching of the injected site is due to itch, but not to pain. The data did not provide support for the idea that histamine produces itch in the mouse.

Keywords: Scratching; Itch; Pain; Compound 48/80; Substance P; Histamine

1. Introduction

Although pruritus is the main and unpleasant symptom of cutaneous diseases and accompanies several visceral disorders, such as chronic renal failure, hepatic cholestasis, and diabetes mellitus, its underlying mechanisms remain unknown. To shed light on the physiological and pathological mechanisms of pruritus, we need animal models of itch. However, there are no reliable animal models available (Woodward et al., 1985). One reason for this might be due to the difficulties in behavioral animal experiments on pruritus. For example, although itch is a sensation that is associated with a strong desire to scratch, the mouse licks, but not scratches, its hind paw when it is subcutaneously (s.c.) injected with the pruritogenic agent compound 48/80 (unpublished observation), a behavioral response similar to that following treatment with the algesiogenic agent formalin (Hunskaar et al., 1985). Pruritus may be a main symptom in canine allergy (Woodward et al., 1985), but intradermal injections of histamine and compound 48/80 cause a painful reaction rather than itching behaviors (Schwartzman, 1965). Humans can distinguish ticklish stimuli from prickly ones, while many mammals respond with a similar stereotypical behavior (see McMahon and Koltzenburg, 1992). In addition, the mouse shows scratching of the ear and body during grooming, which is hard to consider as itch-associated behavior.

Since itch is a subjective sensation and animals do not describe their sensory experiences, for the measurement of itch in animal behavioral experiments, we should use itch-related behaviors that are elicited only by pruritogenic stimuli but not by other sensory stimuli such as painful ones and that are rarely observed in untreated animals. As mentioned above, itch is a sensation that provokes a desire to scratch the stimulated area, probably to scratch away irritants from the skin. Clinically, scratch is used as an objective measure of itch (Savin et al., 1973; Felix and Shuster, 1975; Aoki et al., 1980; Summerfield and Welch, 1980). Therefore, as a first step in developing an animal model of itch, we compared the behavioral effects, especially scratch-eliciting ones, of treatment with pruritogenic and algesiogenic agents in mice. Here, we report that s.c.

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injections of pruritogenic agents, but not algesiogenic ones, into the rostral part of the back produced scratching of the injected site by the hind paws, which probably is an itch-associated behavior.

2. Materials and methods

2.1. Materials

Compound 48/80 (Sigma, St. Louis, USA), substance P (Peptide Institute, Minoh, Japan), histamine (Sigma, St. Louis, USA), and formalin (Wako Pure Chemical Ind., Osaka, Japan) were dissolved in or diluted with physiological saline. Capsaicin (Sigma, St. Louis, USA) was dissolved in physiological saline containing 50% dimethyl sulfoxide. All these agents were s.c. injected into the back at the interscapular level in a volume of 0.10 ml.

2.2. Behavioral experiments

Male ddY mice of 4 weeks of age, weighing 18–23 g, were used in the experiments. They were housed under controlled temperature (23–25°C) and light (lights on from 08:00 to 20:00). Food and water were freely available. Before the experiment, the animals were put into an acrylic cage (22 × 22 × 24 cm) for about 10 min for acclimation. Immediately after s.c. injection, they (generally five animals/cage) were put back into the same cage and, for the observation of scratching, behaviors were recorded using an 8-mm video camera (CCD-700V, Sony, Tokyo, Japan) under unmanned conditions. Scratching of the injected site by the hind paws was counted and that of other sites such as ears was disregarded. Each mouse was used for only one experiment. The mice generally showed several scratches for about 1 s and a series of these behaviors was counted as one incident of scratching at 10-min intervals.

2.3. Data processing

Statistical comparisons were made using one-way analysis of variance and Dunnett's post-hoc test or for data without normal distribution Kruskal-Wallis statistic on ranks and Dunn's post-hoc test; the calculation was done using software SigmaStat (Jandel, San Rafael, USA) and $P < 0.05$ was considered significant. The means of data are presented together with S.E.

3. Results

3.1. Compound 48/80-induced scratching

Compound 48/80 has been shown to produce an itch sensation in humans (Armstrong et al., 1953;

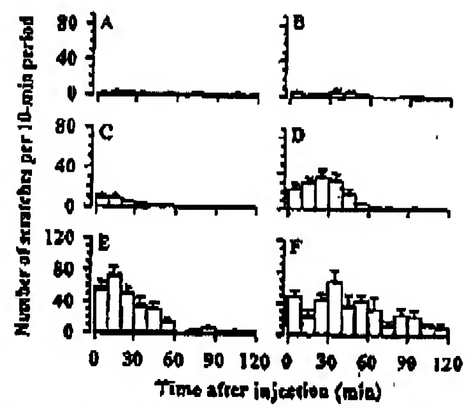

Fig. 1. Time course of scratching after an s.c. injection of compound 48/80. Mice were given an injection of saline (A, $n = 10$) or compound 48/80 at doses of 3 (B, $n = 10$), 10 (C, $n = 20$), 30 (D, $n = 20$), 50 (E, $n = 16$) and 100 μg (F, $n = 10$). Values represent the means and S.E.

Hägermark et al., 1978; Fjellner and Hägermark, 1981). When sixteen mice were given compound 48/80 (50 μg, s.c.) into the rostral part of the back, all showed scratching of the injected site by the hind paws. The first scratching was observed within 5 min after injection in all mice examined and then scratching appeared intermittently. The frequency of behaviors other than scratching, for example grooming and forelimb motions, was not apparently different between compound 48/80- and saline-treated mice.

Fig. 1 shows the time course of scratching behaviors for 2 h after the injection of compound 48/80. When compared with saline, compound 48/80 at doses of 10–100 μg produced apparent scratching, without effects at a dose of 3 μg. The effects of compound 48/80 (10–50 μg) peaked within 30 min and had almost subsided by 60 min, while that of 100 μg of compound 48/80 lasted for more than 60 min. When the number of scratches in 60 min after compound 48/80 was plotted against the dose, the effect was dose-dependent from 10 to 50 μg; the effect of 100 μg was not apparently different from that of 50 μg (Fig. 2).

3.2. Behavioral effects of substance P and histamine

Substance P and histamine as well as compound 48/80 produce an itch sensation in humans (Armstrong et al., 1953; Hägermark et al., 1978; Fjellner and Hägermark, 1981). Injections of substance P at s.c. doses of 100 and 300 μg elicited slight but significant scratching, without effects at lower doses of 10 and 30 μg (Fig. 2). The first scratching after substance P (300 μg) was observed within 5 min in all mice examined ($n = 5$) and the effects of substance P (100 and 300 μg) peaked in the first 10-min period and had almost subsided by 30 min following injection. Grooming and

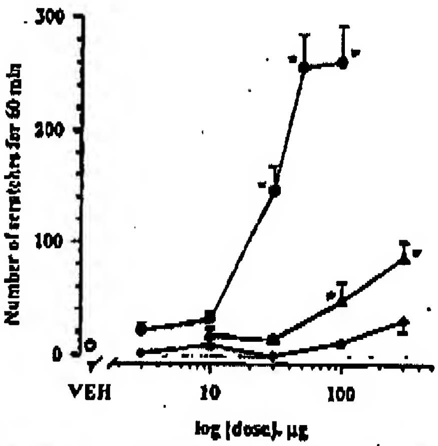

Fig. 2. Dose-response curves for the scratch-inducing effect of compound 48/80, substance P and histamine. Mice were given an s.c. injection of compound 48/80 (●, $n = 10$–20), substance P (▲, $n = 5$–6), histamine (♦, $n = 5$–10) or saline (VEH, ○, $n = 10$). The number of scratches in 60 min was plotted against the dose. Values represent the means and S.E. * $P < 0.05$ when compared with VEH (Dunn's post-hoc test).

forelimb motions were not apparently different between substance P- and saline-treated mice.

Injections of histamine at s.c. doses of 3–300 μg did not have a significant scratching-inducing effect (Fig. 2). Although following the maximum dose tested (300 μg) the number of scratches in 60 min was 31.8 ± 10.5 ($n = 5$), there were few scratches in 20 min following injection. Histamine at the doses tested did not produce any apparent alterations in gross behaviors, such as sedation, and motor functions, such as ataxia and weakness of the hindlimbs.

3.3. Effects of capsaicin and formalin

To determine whether the painful stimulation of the rostral part of the back would elicit scratching behavior, we examined the behavioral effects of s.c. injections of algesiogenic agents, capsaicin and formalin (Fig. 3). In contrast to compound 48/80 and substance P, capsaicin (30 and 100 μg) and dilute formalin (5 mg of formaldehyde) did not induce significant scratching behavior following injection. Although a higher dose (300 μg) of capsaicin resulted in the death of all animals tested ($n = 5$) during the observation period, capsaicin and dilute formalin at the doses tested did not apparently alter gross behaviors and motor functions.

4. Discussion

The main findings of the present study are that, when injected into the rostral part of the back of the mouse, pruritogenic, but not algesiogenic, agents elicited scratching of the injected site by the hind paws. In human subjects, application of 500 μg/ml of compound 48/80 on the blister base (Armstrong et al., 1953), an intradermal injection of 0.3–10 μg/ml (Fjellner and Hägermark, 1981), and an s.c. injection of 10 μg/ml (Hägermark et al., 1978) have been shown to cause an itch sensation the duration of which is relatively short, for example several minutes (Armstrong et al., 1953). In the present experiments, s.c. injections of compound 48/80 at doses of 10–50 μg (100–500 μg/ml) produced scratching behavior in mice in a dose-dependent manner. These concentrations are greater than itch-producing ones in humans and the duration of the scratching was longer than that of the itch sensation in human subjects. Intradermal injections of substance P at doses of 0.1–10 μM (corresponding to about 0.13–13 μg/ml) produce an itch sensation in humans (Hägermark et al., 1978; Fjellner and Hägermark, 1981). Although higher concentrations (1–3 mg/ml) were needed, substance P produced scratching in mice. On the other hand, injections of capsaicin (30 and 100 μg) and dilute formalin (5 mg of formaldehyde) into the rostral back did not elicit scratching behavior. When s.c. injected into the hind paw, these algesiogenic agents elicit licking of the treated paw (Hunskaar et al., 1985; Sakurada et al., 1992), a behavior considered to be a pain-related response. Taken together, these findings suggest that scratching of the treated rostral back by the hind paws is due to itch, but not to pain.

Capsaicin acts on primary afferents to release substance P (for review, see Holzer, 1991), but, in the present experiments, substance P, but not capsaicin, elicited scratching in mice. The precise reason why

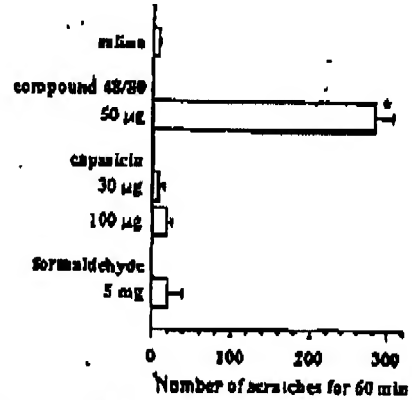

Fig. 3. Scratching following s.c. injections of compound 48/80, capsaicin and formaldehyde. Mice were given an injection of compound 48/80 (50 μg, $n = 19$), capsaicin (30 and 50 μg, $n = 5$ each group), dilute formalin (5 mg of formaldehyde, $n = 5$) or saline ($n = 10$) and the number of scratchings was counted for 60 min. Values represent the means and S.E. * $P < 0.05$ when compared with saline (Dunnett's post-hoc test).

capsaicin did not induce scratching is not clear. However, when applied on the human skin, capsaicin produces a burning pain, but not an itch sensation (Tóth-Kása et al., 1986; Breneman et al., 1992). Taking account of the 'selectivity theory' of neural mechanisms of itch (McMahon and Koltzenburg, 1992), the widespread activation of nociceptive primary afferents with capsaicin might mask the activity of 'itch-signaling' primary afferents induced by released substance P.

Intracranial injections of substance P at relatively low doses induce hindlimb scratching in mice (Dobry et al., 1981) and rats (Van Wimersma Greidanus and Maigret, 1988). However, it is unlikely that peripherally administered substance P acted upon the central nervous system to elicit the scratching. The reasons for this are as follows: first, in the present experiments, substance P at s.c. doses of 100 and 300 μg (corresponding to about 5 and 15 mg/kg, respectively) produced scratching of the injected site in mice, while this peptide at intravenous doses up to 50 mg/kg does not elicit scratching in the same species (Dobry et al., 1981). Second, an intracranial injection of substance P elicits grooming behavior (Dobry et al., 1981; Van Wimersma Greidanus and Maigret, 1988), while an s.c. injection of substance P did not. Third, in our preliminary experiments, substance P (135 μg) injected into the caudal part of the back did not induce scratching of the rostral part of the back. These findings taken together suggest that scratching of the injected site following s.c. injection of substance P is due to a scratch-inducing sensation at the injected skin.

Compound 48/80 and substance P release mediators from the skin mast cells (Barrett et al., 1985; Ebertz et al., 1987; Lowman et al., 1988). Therefore, one of the conceivable mechanisms for the scratch-inducing effects of compound 48/80 and substance P is that these agents act upon the mast cells to release mediators, which produce scratching behavior. Histamine is present in the mast cells and has been thought to be an important mediator of itch. When applied on the blister base at concentrations of 100–1000 $\mu g/ml$ (Armstrong et al., 1953) or intradermally injected at concentrations of 0.3-10 $\mu g/ml$ (Hägermark et al., 1978; Fjellner and Hägermark, 1981), histamine produces an itch sensation in humans. In the present experiments, however, s.c. injections of histamine at doses of 3–300 μg (30–3000 $\mu g/ml$) did not apparently elicit the scratching behavior. Although the precise reason why histamine could not elicit scratching is unclear, one explanation is that, at least in the mouse, histamine is not a strong itch-producing mediator and that scratching induced by compound 48/80 and substance P is mediated by mediators other than histamine. In support of this view, the histamine H_1 receptor antagonist chlorcyclidine inhibits the flare response, but not the itch sensation, induced by an intradermal injection of substance P at a concentration of 10 μM, corresponding to about 13 $\mu g/ml$ (Hägermark et al., 1978). In addition, it is claimed that histamine is not a main mediator of itch in many pruritogenic diseases (Krause and Shuster, 1983; Wahlgren et al., 1990; Hägermark, 1992). Another explanation is that there are species differences in itch mediators and that histamine produces itch in humans but not in mice. In this context, serotonin, another chemical mediator of murine mast cells, apparently elicits scratching in rats (Berendsen and Broekkamp, 1991) and mice (unpublished observation), although it produces mild itch in humans (Fjellner and Hägermark, 1979). In any case, histamine does not induce a desire to scratch in the mouse.

In summary, the present results suggest that scratching of the rostral back injected with compound 48/80 and substance P by the hind paws may be due to itch, an impulse to scratch, but not due to pain. Such pseudo-itch behavior may be a useful index for physiological and pharmacological studies on itch and antipruritic agents. The data did not provide support for the idea that histamine applied peripherally produces itch in the mouse.

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